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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
Neurofibromatosis type2 (NF2) is a familial cancer syndrome that features the development of nervous system tumors. The *NF2*-encoded protein, merlin, localizes to the membrane:cytoskeleton interface, raising the intriguing question of how a protein that occupies such a physical niche controls cell proliferation. To generate an animal model for NF2 and to build the foundation for delineating the molecular function of merlin, we established a *Nf2*-mutant mouse strain through genetic engineerins. $Nf2^{+/-}$ mice develop a spectrum of tumors that is distinct from that of their human counterparts, including osteosarcomas and hepatocellular carcinomas, which exhibit loss of the wild-type *Nf2* allele. Embryos that are homozygous for a null *Nf2* mutation fail to gastrulate, while chimeric embryos partially composed of $Nf2^{-/-}$ cells develop additional defects, including during cardiac development. Together, these observations indicate a requirement for merlin function in several different cell types in the mouse; the study of Nf2 function in these cell types formed the basis of this proposal. The following report describes our accumulating data that indicates an important and general role for *Nf2* function in cell differentiation. In addition, data from these and other studies in our laboratory point to a general role for merlin function in plasma membrane organization in different cell types.

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Introduction:

Mutations in the *NF2* tumor suppressor gene underlie the familial cancer syndrome Neurofibromatosis type 2 (NF2). Human NF2 patients are predisposed to developing schwannomas and meningiomas of the central nervous system. Despite the cloning of the *NF2* gene nearly a decade ago, the molecular function of its encoded protein, merlin is not yet known. Merlin localizes to the membrane-cytoskeleton interface and thus occupies an unusual physical niche for a tumor suppressor. We have generated a *Nf2*-mutant strain of mice that provides the foundation for defining the molecular function of merlin. We have found that $Nf2^{+/-}$ mice, like their human counterparts, are cancer prone but develop several types of tumors that are not features of human NF2. The study of these mice also suggests a role for *Nf2* loss and tumor metastasis. We also found that *Nf2* function is required for several stages of embryonic development. Thus $Nf2^{-/-}$ embryos fail immediately prior to gastrulation due to a defect in the extraembryonic lineage. The study of chimeric embryos that are composed of $Nf2^{-/-}$ and wild-type cells indicated that *Nf2* function is also required for the normal development of several other lineages, including the myocardium and neuroepithelium. Together our studies suggest that *Nf2* function is important in many cell types. The overall goal of this research proposal is to define the signature of *Nf2*-deficiency across several cell types including the ES cell, cardiomyocyte, osteoblast and hepatocyte; each of these cell types is defective when *Nf2*-deficient.

We submitted a progress report one year ago detailing our progress in developing an in vitro system for delineating the function of *Nf2* in cardiomyocyte differentiation. Use of this system revealed that *Nf2*-function is not required generally for the establishment of differentiation programs. Instead, *Nf2*-function is required for the differentiation of specific lineages. Thus although $Nf2^{-/-}$ ES cells can form embryoid bodies that express various markers of differentiation and contain differentiated structures, they cannot differentiate into cardiomyocytes. Similarly, we identified a requirement for *Nf2* function in osteoblast differentiation. Through these studies we also developed an in vitro model of *Nf2;p53*-associated osteosarcoma development. Finally, we established a model for studying *Nf2*-function in the liver in vivo. Following is an update of progress made since that time.

Body

Nf2 function in cardiomyocyte differentiation.

We previously found that chimeric embryos that are composed of $Nf2^{-/-}$;$lacZ^{+}$ and wild-type cells develop striking abnormalities in cardiac morphogenesis. Thus $Nf2^{-/-}$;$lacZ^{+}$ cells that contribute to the developing myocardium invariably form tumor-like lesions in the heart wall. This defect is apparent early during cardiac morphogenesis (by E9.5), suggesting that it may reflect a failure of $Nf2^{-/-}$ cardiomyocyte differentiation. Consistent with this notion, we found that $Nf2^{-/-}$ ES cells cannot differentiate into beating cardiomyocytes in vitro. Our recent studies in fibroblasts have now suggested an exciting explanation for this defect. In other ongoing studies in our laboratory, we have found that the major consequence of *Nf2*-deficiency in primary $Nf2^{-/-}$ mouse embryo fibroblasts (MEFs) is an inability to undergo contact-dependent inhibition of proliferation and to form cadherin-containing adherens junctions. In fact, we found that merlin colocalizes and associates with cadherin-containing adherens junctions in MEFs. As discussed below, we have found that these are also features of $Nf2^{-/-}$ osteoblasts and Schwann cells, and thus appear to be signatures of *Nf2*-deficiency. With this in mind, we have re-examined the phenotype associated with *Nf2*-deficiency in the developing heart. The onset of this phenotype in chimeric embryos roughly coincides with the formation of intercalated discs in wild-type cardiomyocytes; intercalated discs are specialized cell:cell junctions that contain adherens junction components. In fact, Kostetskii et al. (2001), recently generated chimeric embryos that are partly composed of N-*cadherin*-deficient (N-$cad^{-/-}$) and wild-type cells; these embryos develop tumor-like lesions in the developing myocardium that are morphologically identical to those that we observe in $Nf2^{-/-}$;+/+ embryos. We are currently generating additional $Nf2^{-/-}$;$lacZ^{+}$:+/+ embryos and testing the hypothesis that *Nf2*-deficiency leads to defective N-cadherin-mediated cell:cell communication in differentiating cardiomyocytes. These studies will include an ultrastructural comparison of intercalated disc formation in wild-type and $Nf2^{-/-}$ cardiomyocytes.

Nf2 function in osteoblast differentiation.

Both $Nf2^{+/-}$ and compound $Nf2^{+/-}$;$p53^{+/-}$ mice spontaneously develop osteosarcomas, suggesting that *Nf2* function is critical for the control of osteoblast proliferation. In progress described last year, we found that merlin levels and phosphorylation are regulated by cell density and differentiation in wild-type osteoblasts. In addition, we found that primary $Nf2^{+/-}$;$p53^{+/-}$ calvarial osteoblasts grow to a higher density in culture and produce more calcified bone than wild-type cultures. Finally, we found that within a few passages (4-5), these $Nf2^{+/-}$;$p53^{+/-}$ cis cultures exhibit loss of the wild-type *Nf2* and *p53* alleles and become immortalized. Pure $Nf2^{-/-}$;$p53^{-/-}$ cultures can no longer differentiate, do not undergo density-dependent growth arrest and can form tumors when injected subcutaneously into nude mice. Thus we can recapitulate the genetic basis of tumor development in $Nf2^{+/-}$;$p53^{+/-}$ mice in vitro.

In order to define the role of *Nf2* specifically in osteoblast proliferation and differentiation, we obtained $Nf2^{loxP/loxP}$ mice from our collaborator Marco Giovannini (CEPH, Paris). Primary calvarial osteoblasts from $Nf2^{loxP/loxP}$ neonates were cultured and infected with adenoviral expressing the Cre-recombinase (Ad-Cre). Cre expression led to efficient excision of *Nf2* exon 2 and loss of merlin expression. We found that, like $Nf2^{-/-}$;$p53^{-/-}$ osteoblasts, $Nf2^{-/-}$ osteoblasts do not differentiate properly, neither expressing osteocalcin nor producing mineralized bone (Figure 1). Thus *Nf2* function is required for osteoblast differentiation in vitro. It will be important to determine whether *Nf2* function is similarly required for osteoblast differentiation in vivo. To address this question we have obtained *α1coll-Cre* transgenic mice from Dr. Barbara Kream (UConn) and crossed them to $Nf2^{loxP/loxP}$ mice to generate animals with *Nf2*-deficiency in skeletal tissue. The generation and analysis of these animals is underway. Although α1collagen is a marker of the developing skeleton and is expressed at high levels by osteoblasts, these transgenic mice are relatively uncharacterized. To investigate the extent of Cre expression in these mice we have crossed them to a reporter strain (ROSA26-GT) in which Cre-mediated excision of a 'stuffer' fragment will turn on expression of a lacZ transgene, thereby 'reporting' the expression pattern of Cre. The analysis of these mice is underway. In addition, in collaboration with Dr. Phil Hinds (Harvard Medical School), we are generating transgenic mice that express the Cre-recombinase under the control of the bone-specific osteocalcin promoter. Together these studies will allow us to define the function of *Nf2* in osteoblasts in vivo.

Importantly, high cell density and cadherin-mediated cell:cell communication have been shown to be required for osteoblast differentiation. Given our studies in MEFs and cardiomyocytes, we propose that merlin is required for adherens junction formation, contact-dependent growth arrest and subsequent differentiation in osteoblasts. We are currently testing this hypothesis. Preliminary observations indicate that, like MEFs, $Nf2^{-/-}$ osteoblasts do not form adherens junctions. Our goal for the upcoming year is to define the mechanism whereby merlin controls adherens junction formation or stability in both MEFs and osteoblasts; in osteoblasts we will further delineate the link between merlin function and differentiation.

_Nf2_ function in hepatocytes.

Some $Nf2^{+/-}$ mice develop hepatocellular carcinoma that exhibits loss of the wild-type *Nf2* allele; $Nf2^{+/-}$ mice also develop bile duct carcinoma. However, these tumors arise with long latency and their development may be obscured by the frequent development of osteosarcomas in these mice. Moreover, in progress described last year, we found that merlin is associated with hepatocyte membranes and its membrane distribution is regulated by growth factor stimulation. These data suggest that merlin function is important in liver cells.

To study the merlin function in the liver in vivo, we utilized $Nf2^{loxP/loxP}$ mice. These mice were either crossed to mice that express a Cre-recombinase transgene under the control of the liver-specific albumin promoter (*alb-Cre*) or infected with Ad-Cre via tail vein injection, which facilitates viral delivery to the liver. We have now completed an initial

analysis of *Nf2*$^{loxP/loxP}$;*alb-Cre* mice, which exhibit a dramatic phenotype (Figure 2). By 6-8 weeks of age all *Nf2*$^{loxP/loxP}$;*alb-Cre* mice exhibit abdominal swelling; upon dissection it is apparent that this is due to hepatomegaly. In fact, the livers of these mice may constitute 1/4 to 1/3 of the total body weight of the animal. Other organs appear to be unaffected. Histological examination reveals massive infiltration and hyperproliferation in these livers; the cell type affected appears to be either of bile duct or stem (oval) cell origin (Figure 3). Surprisingly, similar lesions are already apparent in the livers of 2-week old mice, suggesting that they may be embryonic in origin. In fact, preliminary results suggest that the administration of Ad-Cre to adult animals followed by partial hepatectomy yields similar lesions. Thus these lesions may reflect uncontrolled proliferation of liver stem cells. These results are exciting for several reasons: 1) They provide the foundation for studying *Nf2* function in the liver in vivo; 2) Genetically manipulated mouse models of liver cancer are rare; 3) The possibility that *Nf2* function is important in liver stem cells has important implications for the study of liver disease in humans; and 4) The notion that merlin function is important in liver cell differentiation is consistent with our studies of merlin function in other cell types as described above. Although liver cancer is not a recognized feature of human NF2, the disease is quite rare (1/40,000 individuals); therefore a variably penetrant associated phenotype may be difficult to recognize. Moreover, examination the NF2 gene in human liver cancers has not been undertaken. In fact, loss of chromosome 22q, where the NF2 locus resides has been detected in several forms of liver cancer.

We are currently examining *Nf2*$^{loxP/loxP}$;*alb-Cre* mice and embryos of various ages to establish the etiology of these lesions. We have gathered a panel of markers that should allow us to define the affected cell type in more detail. Importantly, we have successfully established cell lines from several of these livers, which we can use to examine the cellular and molecular properties of these cells and to examine the effects of merlin reintroduction. For example, given our studies of *Nf2*-deficient MEFs and osteoblasts, it will be interesting to know whether *Nf2*-deficient liver cells form adherens junctions or undergo contact-dependent inhibition of proliferation.

Key research Accomplishments:

- *Nf2*-deficiency leads to loss of contact-dependent inhibition of proliferation and loss of adherens junctions in MEFs. These phenotypes are also features of *Nf2*-deficient osteoblasts and Schwann cells. Re-examination of the phenotype associated with Nf2-deficiency in the developing myocardium in chimeric embryos suggests that this may also reflect failed cadherin-mediated cell:cell attachment.

- *Nf2*-deficiency alone leads to failed osteogenic differentiation. Genetic crosses designed to test the requirement for *Nf2* in osteogenic differentiation/function in vivo are complete and histologic analysis is underway.

- Liver-specific deletion of *Nf2* has been achieved by crossing $Nf2^{loxP/loxP}$ mice with transgenic *alb-Cre* mice. These mice develop hepatomegaly early in life due to the development of invasive tumors that appear to be of stem cell origin. A complete analysis of these tumors is underway; cell lines are easily established from these lesions and provide a valuable tool for defining the cellular and molecular function of *Nf2* in the liver.

Reportable Outcomes:

Some of these data have been incorporated into seminar presentations given by Dr. McClatchey at the following meetings:

November, 2001

Invited seminar: American Cancer Society Harry and Elija Jiler Professors Meeting, San Diego, CA.

January, 2002

Invited speaker: Preclinical Therapeutics for NF meeting, Washington DC.

June, 2002

Invited speaker: Annual National Neurofibromatosis Foundation Consortium meeting, Aspen, CO.

Invited speaker: Mouse Models of Human Cancer Consortium Steering Committee Meeting, Boston, MA.

August, 2002

Invited speaker: 'Cancer' Gordon Conference Meeting, Salve Regina, RI.

A manuscript describing a role for merlin in contact-dependent inhibition and adherens junction formation in MEFs is under review at Genes & Development.

A manuscript describing the characterization of cardiac abnormalities in *Nf2*$^{-/-}$;+/+ chimeric embryos has been prepared and is being submitted.

A manuscript describing the requirement for *Nf2* function in osteogenic differentiation has been prepared.

Conclusions:

The study of Nf2-mutant mice indicates that merlin function is critical in a broad range of cell types. From our efforts to study merlin in several different cell types, two clear themes have emerged: 1) The primary consequence of *Nf2*-deficiency is loss of contact inhibition and loss of stable adherens junctions (this also emerges from our studies of $Nf2^{-/-}$ MEFs); and 2) Merlin plays an important role in controlling programs of cell differentiation in many lineages. In fact, failed differentiation may be a general mechanism whereby loss of *Nf2* promotes tumorigenesis. For example, our studies of chimeric embryos point to a requirement for *Nf2* function in cardiomyocyte differentiation; in the absence of normal differentiation, these cells form tumor-like lesions in the cardiac wall. We now recognize that this phenotype may be due to abnormal cell:cell communication. Similarly, $Nf2^{-/-}$ osteoblasts do not form adherens junctions and do not differentiate. We are currently investigating the link between these cellular phenotypes and *Nf2*-associated osteosarcoma development, which is the hallmark of $Nf2^{+/-}$ mice. These studies have obvious implications for the general study of bone disease in humans. Finally, liver-specific deletion of *Nf2* has yielded an exciting model of primary liver cancer, supporting the idea that merlin functions as a tumor suppressor in this tissue as well. Moreover, preliminary analysis of these tumors suggests that they are of stem cell origin, providing yet another example of a requirement for *Nf2* function in cell differentiation.

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Lallemand D, Curto M, Saotome I, Giovannini M and McClatchey AI. *NF2*-deficiency promotes tumorigenesis and metastasis by destabilizing adherens junctions. Submitted to *Genes & Dev.*

Saotome I, Freiden L and McClatchey AI. The Nf2 tumor suppressor promotes cardiomyocyte differentiation and cell:cell communication. Manuscript in preparation.

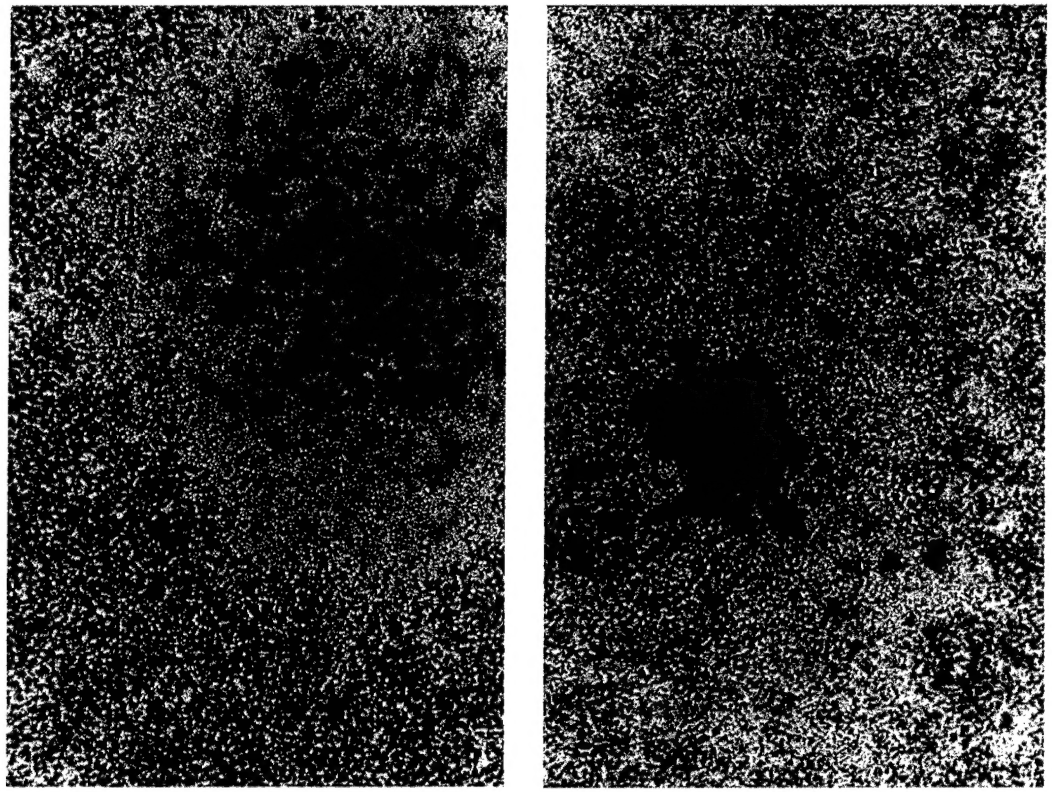

Figure 1: Primary *Nf2*$^{-/-}$ osteoblasts (+Cre) neither form mineralized bone nor express osteocalcin, indicating that *Nf2* function is required for osteoblast differentiation.

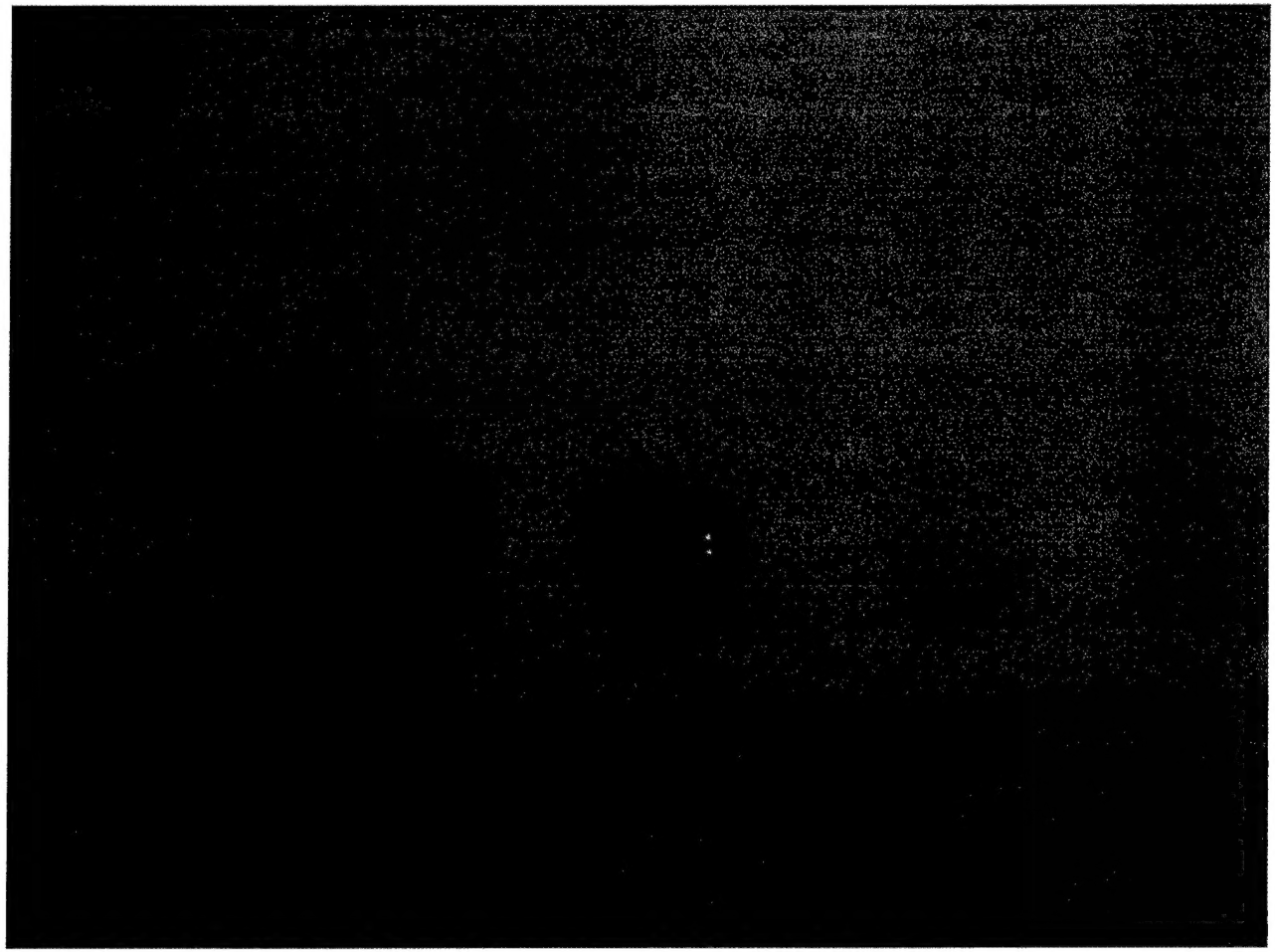

Figure 2: Livers from 6 week old *Nf2*$^{loxP/loxP}$;*alb-Cre* (left) and control (right) mice. Hepatomegaly is apparent in the *Nf2*$^{loxP/loxP}$;*alb-Cre* liver, which can represent as much as 1/3 of the body weight of the mouse at this stage.

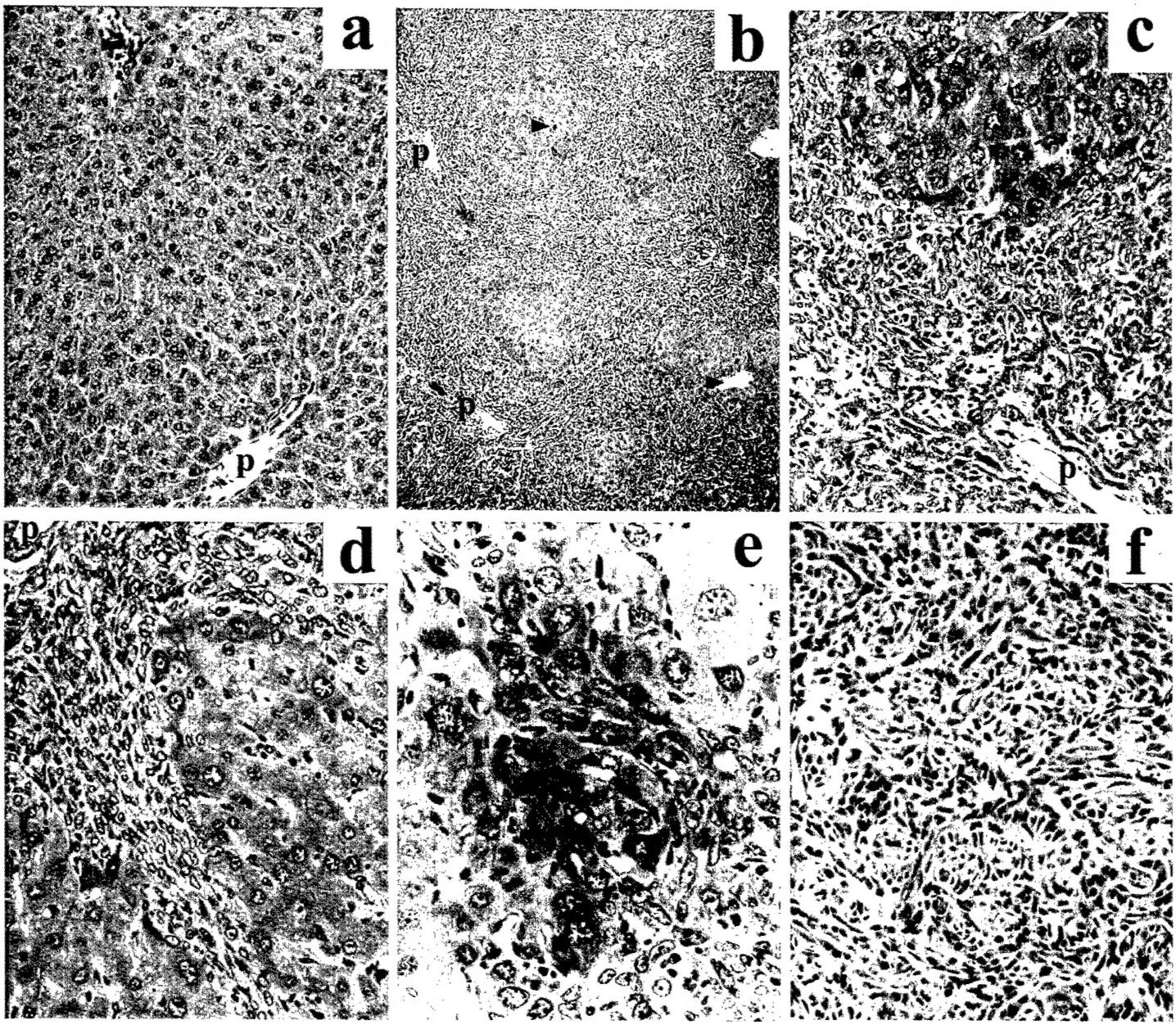

Fig. 3 (H&E staining). Compared to normal liver (**a**), histomorphological examination generally shows intense hyperplasia of bile duct-like cells continuous with the portal tracts (**p**) and infiltrating the surrounding parenchyme along the Zone1 of Rappaport's hepatic acinus (**b**, **c** and **d**). Terminal hepatic veins (arrowheads) are mostly intact, with surrounding hepatocytes appearing normal or with mild regressive aspects (clear, hydropic), often enclosed in pericentral microacini of residual parenchyme (**b**). Cellular morphologies vary from small cells with oblong nuclei, reminiscent of the hepatic stem-like (oval) cells, to polarized cholangiocyte-like cells delimiting incomplete lumina in areas of disorganized neo-ductulogenesis (**c** and **d**). Areas of dysplasia with increased cellular pleomorphism and nuclear atypia become evident within tumors (**e** and **f**).